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14. ABSTRACT

In 30 to 70 % of systemic sclerosis patients, the disease progresses to the lungs and internal organs. This lung fibrosis (i.e. the overexpression of collagen) is the major cause of morbidity and mortality in scleroderma. The overexpression of collagen is accompanied by the overexpression of other extracellular matrix molecules including hyaluronic acid (HA). To evaluate the possibility that HA regulates collagen expression, we treated lung fibroblasts with HA oligomers (fragments of HA that block HA binding to its cell surface receptors CD44, TLR2, and TLR4). Both HA oligomers and lipopolysaccharide (LPS, another TLR2 and TLR4 ligand) had major effects on collagen expression. In addition, HA oligomers affected the expression of the collagen-degrading enzyme MMP-2. These observations open up the possibility that reagents that affect signaling cascades initiated by HA or LPS will have therapeutic value in inhibiting the progression of lung fibrosis in human patients.

15. SUBJECT TERMS
Collagen, hyaluronic acid, lung fibrosis, CD44, TLR2, TLR4

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INTRODUCTION

Systemic scleroderma is a debilitating disease that affects approximately 100,000 people in the US, mostly women, and is characterized by immune system activation, autoimmunity, small-vessel vasculopathy, and skin fibrosis. In 30 to 70 % of patients, the disease progresses to the lungs and other internal organs. Lung fibrosis (i.e. the excessive accumulation of extracellular matrix [ECM] proteins, particularly collagen I) is the major cause of morbidity and mortality in scleroderma. The overexpression of collagen I in fibrotic lung tissue is accompanied by the overexpression of other ECM molecules (e.g. the glycosaminoglycan hyaluronic acid [HA] and the proteins periostin, tenascin-C, and versican) and by inflammatory cell infiltration. In the simplest viewpoint, it is possible that tissue damage leads to inflammatory cell infiltration and that in turn cytokines produced by inflammatory cells and damaged tissue promote the expression of collagen I and other ECM molecules. However, it is more likely that the regulation of these processes (inflammatory cell infiltration, collagen I expression, expression of other ECM molecules) are intertwined in a complex manner. For example, HA is known to promote inflammatory cell infiltration into damaged lung tissue (1) and our Preliminary Studies suggest that it regulates collagen expression by lung fibroblasts. Periostin has been shown to regulate collagen expression in other systems (2). Therefore, our long-term goal is to determine how modulation of the expression and function of ECM molecules such as HA and periostin will alter the progression of lung fibrosis by affecting the expression of collagen I by lung fibroblasts and by affecting inflammatory cell infiltration. These experiments will be performed in both in vitro and in vivo model systems. In vitro experiments will be performed using cultured lung fibroblasts. In vivo experiments will be performed using mice in which lung fibrosis is induced by intratracheal delivery of bleomycin. While bleomycin may not be a perfect model for human diseases, it is a very convenient and reliable way of inducing lung fibrosis in mice and is the best available model for scleroderma lung disease because both bleomycin treatment and scleroderma lung disease produce inflammatory alveolitis and overproduction of collagen and other ECM molecules.

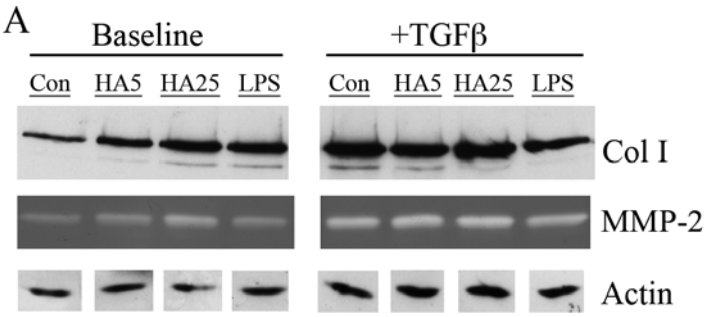
BODY

Our research accomplishments in the first year of the project were limited by time lost due to a change in Principal Investigator (PI). Little more than 2 months was available to perform research after the change was completed. Fortunately, essentially no funds were spent prior to the change in PI. Therefore, we are pleased with the progress that we have made in this short time period and we are confident that we have sufficient resources available to complete the research described in the Work Plan within the three year tenure of this grant.

HA is composed of long chains of repeating N-acetyl-glucosamine-D-glucuronic acid disaccharides that can easily reach molecular weight of > than 1×10^6 daltons. HA binds to other extracellular matrix molecules and to cell surface receptors including CD44, TLR2, and TLR4 (1). The binding of HA to proteins can be inhibited by the presence of HA oligomers (short HA segments with a molecular weight of about 2×10^3 daltons (3). In the Preliminary Studies reported in the grant proposal leading to this project, using primary cultures of normal adult human lung fibroblasts (NLF), we observed that HA oligomers inhibited collagen expression. Because the use of these primary cultures is sometimes criticized due to variations between strains isolated from different individuals, we chose to initiate our experiments using the human embryonic lung fibroblast cell line MRC-5. Experiments were performed in the absence of added TGFβ to simulate baseline conditions in normal lung tissue and in the presence of added TGFβ to simulate the conditions in fibrotic lung tissue where high levels of TGFβ are present and promote the expression of ECM molecules.

As in our experiments using NLF, the addition of HA oligomers had a profound effect on collagen expression by MRC-5 cells. However, whereas HA oligomers inhibited the baseline expression of collagen by NLF, they promoted the baseline expression of collagen by MRC-5 cells three-fold (Fig. 1). Interestingly, HA oligomers had no effect or possibly a small inhibitory effect on TGFβ-stimulated collagen expression by MRC-5 cells. While the data shown in Fig. 1 was obtained using cells incubated in the presence of HA oligomers for 48 hours, these effects were already apparent following a 6-hour incubation. In summary, given that the use of HA oligomers to block HA signaling promotes collagen expression by MRC-5 cells in the absence of added TGFβ and has little effect on collagen expression by cells treated with TGFβ, these results suggest that HA itself inhibits collagen expression by MRC-5 cells in the absence of TGFβ and has little effect on collagen expression by MRC-5 cells in the presence of TGFβ.

A



B

		Col I	MMP-2
Baseline	Con	1	1
	HA5	1.7	1.0
	HA25	3.1	2.1
	LPS	2.4	0.7
+TGFβ	Con	2.6	1.5
	HA5	2.0	1.6
	HA25	2.1	1.7
	LPS	1.6	1.9

Figure 1. Effects of HA Oligomers and LPS on Collagen and MMP-2 Expression by MRC-5 Cells. Cell cultures were initiated by passaging 2 x 10^5 cells into each well of several 6-well plates. After 24 hours, serum-containing medium was replaced with serum-free medium supplemented with no addition (Con), 5 micrograms per ml of HA oligomers (HA5), 25 micrograms per ml of HA oligomers (HA25), or 0.5 micrograms per ml of lipopolysaccharide (LPS) in the presence or absence of 4 nanograms per ml of TGFβ. After 48 h, the medium and cell layer were harvested. **(A) Detection of collagen I, MMP-2 and actin.** Collagen in the medium and actin (loading control) in the cell layer were detected by Western blotting. MMP-2 in the medium was detected by gelatin zymography. Collagen and MMP-2 in the cell layer are not shown because the level of these proteins in the cell layer was < 10 % of their level in the medium. **(B) Quantification of A.** The relative level of each protein in each sample was quantified by densitometry. The levels of collagen and MMP-2 in each sample were normalized against the actin levels, the normalized level of collagen and MMP-2 in the Baseline sample (i.e. no added TGFβ) with no addition was defined as 1 arbitrary unit. The data presented here are representative results from one of three experiments in which similar results were obtained. HA oligomers were Catalog Number HYA-OLIGO10EF-1 from Hyalose (Oklahoma City, OK). LPS was Product Number L 4391 from Sigma (St. Louis, MO).

While TLR2 and TLR4 serve as cell surface HA receptors, they are better known as LPS receptors (1). Therefore, we also examined the effect of LPS on collagen expression by MRC-5 cells. Interestingly, like HA oligomers, LPS promoted the baseline expression of collagen (Fig. 1). However, unlike HA oligomers, LPS inhibited TGFβ-induced collagen expression (Fig. 1). The level of collagen in tissues is dependent both on the expression of collagen and the degradation of collagen by MMPs and other enzymes. Therefore we examined the effects of HA oligomers and LPS on the expression of MMP-2. HA oligomers increased the baseline expression of MMP-2, but had little or no effect on TGFβ-induced MMP-2 expression (Fig. 1). LPS had relatively little effect on MMP-2 expression in the presence or absence or TGFβ. Our results are summarized in Table 1. It is noteworthy that the inhibitory effect of HA on collagen expression may be blunted by its inhibitory effect on collagen degradation mediated by MMP-2.

Table 1. Effects of HA and LPS on Col I and MMP-2 Expression by MRC-5 Lung Fibroblasts

	Baseline (No TGFβ)		+ TGFβ	
	HA	LPS	HA	LPS
Col I	Inhibits	Promotes	Little Effect	Inhibits
MMP-2	Inhibits	Little Effect	Little Effect	Little Effect

Because HA oligomers inhibit the function of HA (3), we assume that the function of HA is opposite to the effects that we observed with HA oligomers. Changes of 30 % or less were considered to be "Little Effect".

The results of these experiments and the possible discrepancy between results obtained with NLF and MRC-5 cells highlights the importance of repeating these experiments with NLF and with lung fibroblasts derived from the fibrotic lung tissue of scleroderma patients (SLF). If MRC-5 cells differ from NLF and if MRC-5 cells treated with TGFβ differ from SLF, it will become all the more important to evaluate how these various cell types differ in their expression of HA, CD44, TLR2, and TLR4 and to determine how modulating the expression or function of HA and its receptors affects collagen expression in these cells. A particularly attractive approach to this problem would be to isolate lung fibroblasts from control mice and knockout mice lacking CD44, TLR2, and TLR4.

KEY RESEARCH ACCOMPLISHMENTS

We have observed that:

- HA oligomers promote baseline collagen expression in MRC-5 cells
- LPS promotes baseline collagen expression in MRC-5 cells
- HA oligomers promote baseline MMP-2 expression in MRC-5 cells
- LPS inhibits TGFβ-induced collagen expression in MRC-5 cells

REPORTABLE OUTCOMES

None.

CONCLUSION

The observation that HA oligomers affect the expression of collagen and MMP-2 by lung fibroblasts supports the idea that the expression of one ECM molecule can regulate the expression of another ECM molecule and raises the possibility of using fragments of ECM

molecules or inhibitors or activators of ECM molecule-initiated signaling pathways as therapeutic agents. The possibility that HA oligomers may promote collagen expression in some types of lung fibroblasts while inhibiting collagen expression in other types of lung fibroblasts suggests many ways in which our Work Plan needs to be refined. It will be critical in various cell types to compare both the baseline and TGFβ-induced levels of expression of HA and the HA receptors CD44, TLR2, and TLR4 and to use several different approaches to determine how modulating the expression or function of HA and its receptors affects collagen expression. A particularly attractive approach to this problem would be to isolate lung fibroblasts from control mice and knockout mice lacking CD44, TLR2, and TLR4. In summary, our observations that HA oligomers and LPS can regulate collagen expression in lung fibroblasts opens up the possibility that reagents that activate or block the signaling cascades initiated by HA or LPS will have therapeutic value in inhibiting the progression of lung fibrosis in human patients.

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APPENDICES

None.